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Transcriptional activation of bacteriophage λ DNA replication *in vitro*: regulatory role of histone-like protein HU of *Escherichia coli*

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Initiation of bacteriophage λ DNA replication *in vivo* and in crude *in vitro* systems is strongly dependent on transcription at or near the λ replication origin (*oriλ*). Through its capacity to prevent RNA polymerase-mediated 'transcriptional activation' of λ DNA replication, the λ *cI* repressor is capable of negatively regulating initiation of λ DNA replication, even when all required replication proteins are present. Surprisingly, the strict requirement for transcriptional activation of λ DNA replication was lost when λ replication was initiated in an *in vitro* system composed of nine purified replication proteins [Mensa-Wilmot *et al.* (1989) *J. Biol. Chem.*, 264, 2853-2861]. We have found that crude extracts of *Escherichia coli* contain proteins that are capable of restoring the physiological linkage between transcription and *oriλ*-dependent replication when they are added to the nine-protein replication system. The protein primarily responsible for this effect has been purified and identified as protein HU, a histone-like protein that is a major constituent of the bacterial nucleoid. HU, when present at a 1:1 weight ratio with supercoiled *oriλ* plasmid, is a potent inhibitor of λ DNA replication in the nine-protein replication system. However, when the *oriλ* template is transcribed by *E.coli* RNA polymerase, the HU-mediated inhibition of λ DNA replication is abolished. HU does not inhibit propagation of λ replication forks. Instead, HU apparently interferes with the assembly or function of nucleoprotein structures containing the *E.coli* DnaB helicase that are formed at *oriλ* prior to priming and DNA synthesis. We suggest that the chromatin structure of the template DNA in the region surrounding *oriλ* plays a central role in the negative regulation of the initiation of λ DNA replication *in vivo*.

Key words: bacteriophage λ/DNA replication/histone-like proteins/HU protein/transcriptional activation

Introduction

One of the most strictly regulated biological processes is the initiation of chromosomal DNA replication. However, little is known about the molecular basis for this stringent control of cellular DNA replication, largely because of the enormous complexity of the process. The factors that regulate initiation frequency have been most thoroughly characterized in *Escherichia coli*. Genetic and physiological studies suggest that transcription at or near the *E.coli* replication origin (*oriC*) is both essential and rate-limiting for initiation (see von Meyenburg and Hansen, 1987; McMacken *et al.*, 1987, for recent reviews).

To learn more about the fundamental features of the regulation of chromosomal DNA replication, our laboratory has been examining the biochemical events involved in the initiation of bacteriophage λ DNA replication. One compelling factor underlying our selection of this temperate coliphage as a model for DNA replication is the well-documented sensitivity of λ DNA replication *in vivo* to phage repressor proteins. The λ *cI* repressor is capable of directly blocking the initiation of λ DNA replication, even when all required phage and host-encoded replication proteins are present in the cell (Thomas and Bertani, 1964; Green *et al.*, 1967). This epistatic action of the λ repressor was initially surprising, since more than 1000 bp separate the closest known repressor-binding site, o_R from the λ replication origin (*oriλ*) (Figure 1).

The initial clues that led to the currently accepted model for repressor action in λ DNA replication came from studies of the properties of λ mutants (termed ri^c mutants) that had acquired the capacity to replicate in the presence of the λ repressor (Dove *et al.*, 1969). Every λri^c phage was found to contain a new repressor-insensitive promoter (e.g. λ*c*17, Figure 1). Furthermore, each newly created promoter

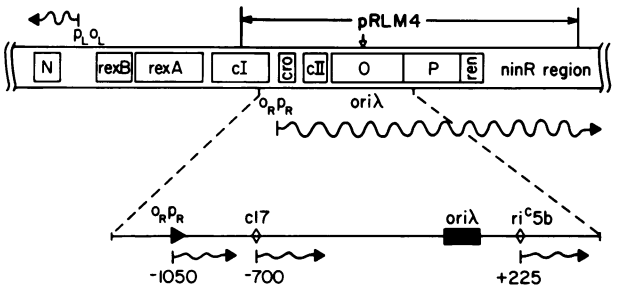

Fig. 1. Genetic and transcriptional map of the region surrounding the λ replication origin with locations of promoters that can serve to transcriptionally activate λ DNA replication. (Upper) The genetic map of the λ chromosome between nucleotide positions 35 000 and 42 000 (Sanger *et al.*, 1982) is depicted. The position of the λ replication origin (*oriλ*) is denoted with a downward arrow. The wavy lines indicate the regions transcribed from the primary promoters (p_R, p_L) used during vegetative growth of the virus. The portion of the λ chromosome contained in the *oriλ* plasmid (pRLM4), used as the template for *in vitro* replication in this study, is indicated. (Lower) An expanded map of the region around the λ replication origin. The positions of promoters in wild type or mutant λ that can direct transcriptional activation of λ DNA replication are shown. The wavy lines depict the direction of transcription that is initiated at each promoter. The numbers below each promoter indicate the approximate number of base pairs from the start point of transcription to the center of the four tandem λ O protein recognition sites located in *oriλ*.

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directed transcription across or near the *ori*λ region. Based on these and related studies, Dove and colleagues proposed that the λ replication origin is maintained in a quiescent state in the cell until it is actively transcribed (Dove *et al.*, 1969). The repressor-sensitive p_R promoter is the only promoter capable of directing transcription across *ori*λ in a wild-type phage (Figure 1, top). Inhibition of the p_R promoter by the λ repressor would, therefore, also block the initiation of λ DNA replication by preventing 'transcriptional activation' of the λ replication origin. The transcriptional activation model was modified slightly after later studies demonstrated that some of the ri^c promoters mapped at least 115 bp to the right of any essential *ori*λ sequence elements and directed transcription away from the λ replication origin (Furth *et al.*, 1982) (e.g. ri^c5b, Figure 1). Thus, transcription of the λ replication origin *per se* is not required for the initiation of λ DNA replication *in vivo*. Transcription of the region to the right of *ori*λ affords the requisite transcriptional activation.

Crude soluble enzyme systems have been developed that support the specific initiation of DNA replication at a λ replication origin, when the origin is present on a negatively supercoiled plasmid template (*ori*λ plasmids) (Anderl and Klein, 1982; Tsurimoto and Matsubara, 1982; Wold *et al.*, 1982). The available evidence suggests that initiation of λ DNA replication in these crude *in vitro* systems is, as *in vivo*, obligatorily coupled to transcriptional activation. Rifampicin, a specific inhibitor of *E.coli* RNA polymerase, is a potent inhibitor of λ DNA replication in such crude systems (Anderl and Klein, 1982; Tsurimoto and Matsubara, 1982; Wold *et al.*, 1982). Moreover, addition of physiological levels of purified λ repressor to the *in vitro* replication system specifically suppresses initiation of λ DNA replication (McMacken *et al.*, 1983; M.Wold and R.McMacken, unpublished data). Yet, if the *ori*λ plasmid template utilized in the crude *in vitro* system also carries the repressor-insensitive *c*17 promoter (Figure 1), then initiation of λ DNA replication proceeds normally in the presence of high levels of λ repressor (McMacken *et al.*, 1983).

We have recently reported (Mensa-Wilmot *et al.*, 1989) the development of a minimal *in vitro* system, composed of nine purified λ and *E.coli* proteins, that specifically replicates *ori*λ plasmids. The required proteins include two λ-encoded replication initiators (the λ O and P proteins), five *E.coli* proteins that participate in propagation of replication forks along the bacterial chromosome (DnaB helicase, DnaG primase, single-stranded DNA binding protein, DNA polymerase III holoenzyme and DNA gyrase), and two *E.coli* heat-shock proteins (DnaJ and DnaK proteins). For the most part, the properties of λ DNA replication in the purified protein system are physiological. We discovered one surprising exception, however. *Ori*λ-specific DNA replication can be initiated in the absence of transcriptional activation in the minimal system (Mensa-Wilmot *et al.*, 1989). Given the physiological nature of this multienzyme λ replication system in other respects, we presume that the lack of a requirement for *E.coli* RNA polymerase transcription is not an artifact of the *in vitro* system.

As a first step toward an understanding of the molecular basis of transcriptional activation of λ DNA replication, we sought to identify putative *E.coli* proteins that could play a regulatory role in this process. We report here the isolation of a bacterial protein that directly blocks the initiation of λ DNA replication *in vitro*. This cellular inhibitor appears to be the histone-like *E.coli* HU protein. Moreover, when either this inhibitor or purified HU is added to the minimal λ replication system, this nine-protein system apparently acquires the capacity to physiologically regulate the initiation of λ DNA replication, inasmuch as the initiation process becomes strictly dependent on RNA polymerase transcription of the template DNA. A preliminary analysis suggests that the presence of HU in the *in vitro* system interferes with the assembly or function of one or more of the nucleoprotein structures that are formed at *ori*λ prior to priming and the initiation of DNA replication.

Results

Isolation of an E.coli protein that blocks the initiation of bacteriophage λ DNA replication

Initiation of bacteriophage λ DNA replication *in vivo* and in crude cellular extracts requires transcription at or near *ori*λ by *E.coli* RNA polymerase. This strict physiological dependence of λ DNA replication on transcription was not observed, however, when the λ initiation process was reconstituted *in vitro* with nine purified proteins (Mensa-Wilmot *et al.*, 1989). To explain this discrepancy, we surmised that phage λ DNA replication *in vivo* is controlled by *E.coli* regulatory proteins whose inhibitory effect could be counteracted by transcription of the λ origin region by RNA polymerase. Since the reconstituted λ replication system is composed of highly purified replication proteins, it presumably does not contain significant quantities of the putative regulatory protein. Initiation of λ DNA replication in this system, accordingly, would proceed in the absence of added RNA polymerase.

To facilitate a search for a bacterial protein that blocks initiation of λ DNA replication, but only under conditions where there is no transcription near the λ origin, we devised a combination assay (see Materials and methods). First, we determined if a particular protein fraction could inhibit the initiation of λ DNA replication when it was added to the reconstituted (RNA polymerase-free) λ replication system. Next, in a separate assay, we assessed the possible physiological relevance of any inhibition that was observed. An inhibitory protein fraction was considered to contain candidate regulatory proteins if its inhibitory effect on λ DNA replication could be partially or completely negated when the λ replication system was also supplemented with purified *E.coli* RNA polymerase and ribonucleoside triphosphates (rNTPs).

A preliminary analysis indicated that crude extracts of *E.coli* contained potential regulatory activity and that this activity was most potent in fractions that were rich in cellular DNA. Based on this information, a fractionation protocol was devised (see Materials and methods) that enabled the isolation of a bacterial protein that specifically inhibits the initiation of λ DNA replication *in vitro*. The most highly purified preparation of this protein, which we have named the λ replication inhibitor, contains a major polypeptide of M_r 10 000 (Figure 2). The crude fractions generated early in the purification protocol contained additional inhibitors of λ DNA replication, most of which apparently act nonspecifically (e.g. nucleases and topoisomerases which relax the supercoiled template). Therefore, it was not

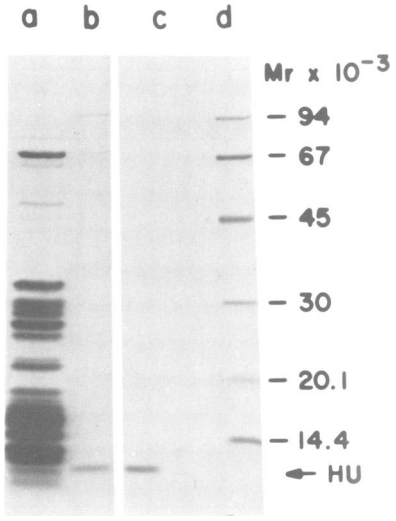

Fig. 2. Polyacrylamide gel electrophoretic analysis of the purified inhibitor of λ DNA replication. Protein fractions (see Materials and methods) containing activity that inhibited λ DNA replication *in vitro* were analyzed by electrophoresis in a polyacrylamide gradient gel in the presence of SDS as detailed in Materials and methods. Samples were: **lane a**, λ replication inhibitor (76 μg of Fraction IV); **lane b**, λ replication inhibitor (1.5 μg of Fraction V); **lane c**, HU protein (2 μg); **lane d**, molecular weight markers.

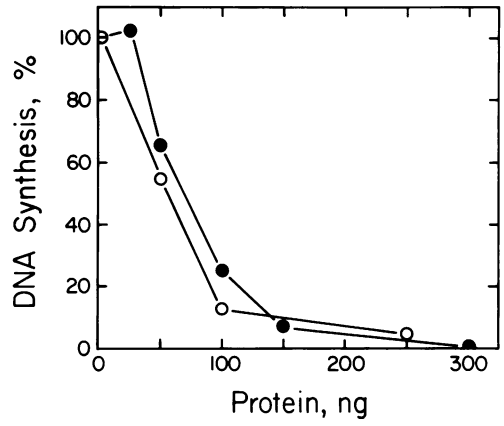

Fig. 3. HU protein inhibits *ori*λ plasmid DNA replication *in vitro*. Standard *ori*λ plasmid replication mixtures (Mensa-Wilmot *et al.*, 1989) were assembled on ice and supplemented with the indicated amounts of HU protein (closed circles) or purified λ replication inhibitor (Fraction V; open circles). DNA synthesis was measured after a 30 min incubation at 30°C. 100% DNA synthesis represents incorporation of 350 pmol of labeled deoxynucleotide into acid-insoluble material. The data presented represent the averages of duplicate determinations.

possible to determine accurately the yield of the putative regulatory protein.

The λ replication inhibitor is the E.coli HU protein

Based on the polypeptide size of the λ replication inhibitor and on its capacity to bind to double-stranded DNA–cellulose, we suspected that the inhibitor might be identical to *E.coli* protein HU. HU, a major component of the bacterial nucleoid, is a histone-like protein that forms nucleosomal structures on duplex DNA (Drlica and Rouvière-Yaniv, 1987). The identification of the purified inhibitor as the bacterial HU protein was confirmed by three separate experimental observations. First, the predominant

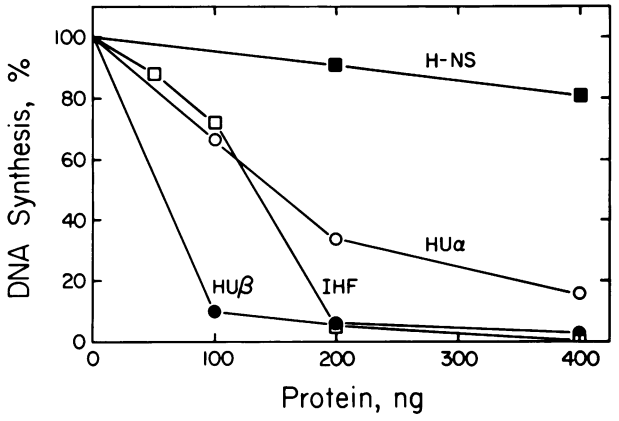

Fig. 4. Inhibition of λ DNA replication by histone-like proteins. The effect of different histone-like proteins on *ori*λ plasmid DNA replication was determined as described in the legend to Figure 3. 100% DNA synthesis represents the incorporation of 440 pmol of labeled deoxynucleotide into acid-insoluble material. HUα subunit, open circles; HUβ subunit, closed circles; integration host factor (IHF), open squares; HNS protein, closed squares.

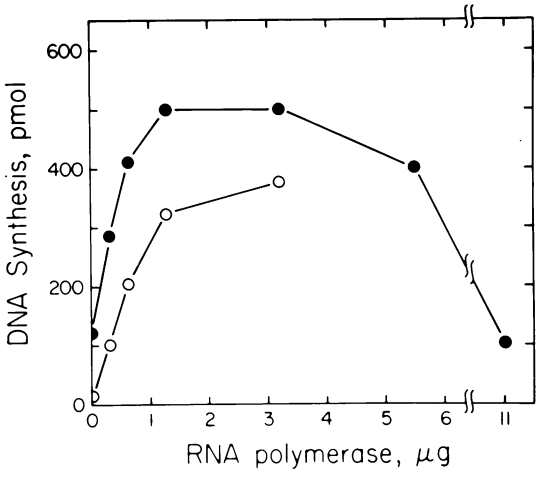

Fig. 5. Determination of the amount of RNA polymerase required to reverse HU-mediated inhibition of λ DNA replication. The standard *ori*λ replication mixture was assembled on ice and supplemented with rCTP, rGTP and rUTP (each at 500 μM final concentration) and with HU protein (100 ng, closed circles; 200 ng, open circles). RNA polymerase was added as indicated. The amount of DNA synthesis was measured after a 60 min incubation at 30°C. All points represent the average of duplicate determinations. In the absence of HU and RNA polymerase, 690 pmol of DNA synthesis was obtained.

polypeptide in our preparation of the λ DNA replication inhibitor was found to co-migrate with authentic HU protein during SDS–PAGE (Figure 2). Second, purified HU protein also inhibited the replication of *ori*λ plasmid DNA templates in the reconstituted λ replication system (Figure 3). Moreover, the specific inhibitory activities of HU and the λ replication inhibitor were similar (Figure 3). Finally, as assayed by immunoblotting, antibodies directed against purified HU or directed against the HUβ subunit each specifically recognized the λ replication inhibitor (data not shown).

Native HU protein is predominantly a heterotypic αβ-dimer, but HU preparations also contain small amounts of α_2 and β_2 homodimers (Rouvière-Yaniv and Kjeldgaard, 1979). Our tests of the purified β subunit of HU indicated that it was as potent an inhibitor of λ DNA replication as

Table I. RNA polymerase transcription is required for replication of HU-coated *ori*λ DNA

Additions	DNA synthesis (pmol)
1 None	520
2 HU	77
3 HU, RNAP[a]	25
4 HU, RNAP, rNTPs	280
5 HU, RNAP, rNTPs, rif	22
6 RNAP	160
7 RNAP, rNTPs	380
8 RNAP, rNTPs, rif	170

The standard *ori*λ replication reaction described in Materials and methods was modified to contain HU protein (105 ng), RNA polymerase (1.3 μg), rNTPS (CTP, GTP and UTP, each at 500 μM) and rifampicin (30 μg/ml), as indicated. DNA synthesis was measured after a 90 min incubation at 30°C.
[a]Abbreviations: RNAP, RNA polymerase; rif, rifampicin.

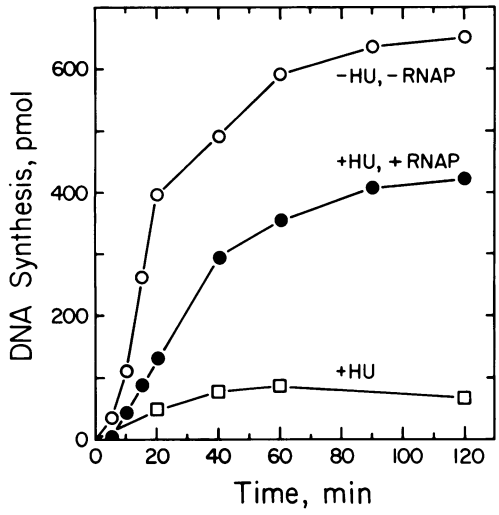

Fig. 6. Time course of λ DNA replication in the presence and absence of HU and RNA polymerase. Standard *ori*λ replication reaction mixtures were assembled at 0°C and supplemented with rNTPs (500 μM final concentration of each CTP, GTP and UTP) and, where indicated, with HU protein (100 ng). After a 10 min incubation on ice, RNA polymerase (RNAP; 1.6 μg) was added, as indicated (closed circles), and all reaction mixtures were incubated at 30°C. DNA synthesis was measured at the indicated times after the start of incubation at 30°C.

the native HU heterodimer (Figure 4). The HUα subunit, though, was considerably less active.

Is the inhibitory effect of HU on λ DNA replication specific or do other histone-like proteins act in a similar fashion? Another protein that may play a role in the compaction of the bacterial chromosome is the HNS protein (M_r 16 000) (Lammi *et al.*, 1984). This protein, which is apparently equivalent to protein H1 (Spassky *et al.*, 1984; Varshavsky *et al.*, 1977; Drlica and Rouvière-Yaniv, 1987), binds tightly and nonspecifically to duplex DNA and is present at moderately high concentrations in *E.coli* cells. In contrast to the strongly inhibitory effect of HU protein on λ DNA replication, HNS caused only a slight reduction in λ DNA synthesis (Figure 4) and did not augment HU-mediated inhibition (data not shown).

The integration host factor (IHF) of *E.coli*, a histone-like, sequence-specific DNA binding protein, shares considerable

Table II. Requirements for λ DNA replication in the presence of HU protein

Component omitted	DNA synthesis (pmol)
None	340
λ O	3
λ P	0
DnaB	5
DnaJ	41
DnaK	0
SSB	0
primase	41
DNA polymerase III holoenzyme	1
DNA gyrase	0
RNA polymerase	41
HU	310
None[a]	50

The standard *ori*λ replication reaction was modified to contain CTP, GTP and UTP (500 μM each), HU protein (100 ng) and RNA polymerase (1.6 μg). Individual components were omitted as indicated. DNA synthesis was measured after a 90 min incubation at 30°C. Each result is the average of duplicate measurements.
[a]Rifampicin was present at 20 μg/ml.

Table III. Template specificity of DNA replication in the presence of HU protein and RNA polymerase

Template	Origin	DNA synthesis (pmol)
pRLM4	λ	340
pRLM5	phage 82	68
M13mp8 RF[a]	M13	74
M13*oriC*26 RF	*E.coli*	10

The standard *ori*λ replication reaction was modified to contain HU protein (105 ng), RNA polymerase (1.6 μg), and rNTPs (CTP, GTP and UTP, each at 500 μM). The DNA template (215 ng) used was varied as indicated. DNA synthesis was measured after a 90 min incubation at 30°C.
[a]RF, replicative form.

sequence homology with HU (Drlica and Rouvière-Yaniv, 1987). IHF aids the establishment of lysogeny by bacteriophage λ by functioning directly in integrative recombination (Nash and Robertson, 1981) and by indirectly stimulating the expression of the λ repressor (Drlica and Rouvière-Yaniv, 1987). Because IHF is evolutionarily related to HU, we tested whether IHF might function in yet a third manner to promote lysogeny, by inhibiting the initiation of λ DNA replication. We found that IHF, like HU, inhibited the *in vitro* replication of *ori*λ DNA (Figure 4).

Transcription of oriλ DNA by E.coli RNA polymerase counteracts inhibition of λ DNA replication by HU protein

A fundamental requirement of the assay utilized in the purification of the λ replication inhibitor was that the inhibition of λ DNA replication be sensitive to transcription by RNA polymerase. It is not surprising, then, that HU-mediated inhibition of *in vitro* λ DNA replication was also nullified by the presence of RNA polymerase and rNTPs in the standard λ replication reaction mixture (Figure 5). However, the level of RNA polymerase required to alleviate inhibition by HU must be determined for each reaction

condition, since RNA polymerase itself can inhibit λ DNA replication when present in excess (Figure 5).

The mere presence of RNA polymerase in the reaction mixture is not sufficient to overcome the block to λ DNA replication caused by HU (Table I, line 3). Instead, activation of DNA replication in the presence of HU apparently requires RNA synthesis by RNA polymerase. Only limited amounts of λ DNA synthesis were obtained when one or more of the rNTPs were omitted from a coupled transcription−replication reaction mixture (Table I and unpublished data) or when this *in vitro* system was supplemented with rifampicin (Table I), which blocks the first translocation step during transcription by *E.coli* RNA polymerase. We also found that transcription largely relieved the inhibition of λ DNA replication mediated by IHF (data not shown).

We tentatively conclude from these results that it is transcription *per se*, as opposed to the mere physical presence of this enzyme, that counteracts HU-mediated suppression of λ DNA replication. A convincing demonstration of this point, however, is complicated by the fact that RNA polymerase itself partially inhibits λ DNA replication (Figure 5 and Table I, line 7). This inhibition becomes considerably more potent when transcription is blocked (Table I, lines 6 and 8). Inhibitory effects of *E.coli* RNA polymerase on DNA replication have also been described in an *in vitro* system for bacteriophage T4 DNA replication (Bedinger *et al.*, 1983). In the T4 system, stationary RNA polymerase molecules that are tightly bound to the template DNA act as barriers to replication fork movement. RNA polymerase apparently behaves in a similar fashion to obstruct λ DNA replication, inasmuch as the block to DNA synthesis was greatly diminished when the coupled system was supplemented with rNTPs (Table I, line 7). In the presence of rNTPs, RNA polymerase forms mobile transcription complexes that apparently are considerably less inhibitory to the propagation of λ DNA replication forks than are stationary complexes. The residual inhibition mediated by RNA polymerase in the presence of rNTPs may partially account for the inability to transcriptionally activate all of the HU-coated *ori*λ plasmid template (Table I; see also Figure 6).

*Properties of ori*λ *plasmid DNA replication in the presence of HU protein and RNA polymerase*

We examined how the addition of HU protein and RNA polymerase to the minimal nine-protein system for λ DNA replication (Mensa-Wilmot *et al.*, 1989) affected the replication properties of *ori*λ plasmids. The presence of these auxiliary proteins caused the initiation of λ DNA replication to be delayed by ~5 min, and the rate and extent of DNA synthesis were noticeably reduced (Figure 6). However, except for a requirement for RNA polymerase, the proteins required for replication of *ori*λ plasmids in the presence of inhibitory levels of HU (Table II) were the same nine proteins as those needed to establish the minimal λ replication system (Mensa-Wilmot *et al.*, 1989).

As analyzed by gel electrophoresis, the *ori*λ plasmid replication intermediates and products synthesized in the coupled transcription−replication system in the presence of HU were nearly identical to those produced in the minimal λ replication system (Mensa-Wilmot *et al.*, 1989). The most obvious difference between the replication pathways of *ori*λ plasmids in the two systems was that multiply-intertwined daughter chromosomes were not as predominant an intermediate in the coupled transcription-replication system (data not shown).

A substantial reduction in the origin specificity of DNA synthesis (Table III) (Mensa-Wilmot *et al.*, 1989) was the only significant alteration produced by the addition of HU and RNA polymerase to the standard λ replication system. The generation of adventitious RNA primers by RNA polymerase transcription could acount for the partial loss of origin specificity. It is interesting that the presence of the histone-like HU protein is reported to improve the origin specificity of an *in vitro* system that is designed to replicate plasmids containing the *E.coli* origin of replication, *oriC* (Dixon and Kornberg, 1984). In the minimal λ DNA replication system, stringent replication specificity is partially lost when transcriptionally active RNA polymerase is present (Mensa-Wilmot *et al.*, 1989). Complete replication specificity is not recovered, however, when HU is also present in the system (Table III).

Effect of λ *repressor on* λ *DNA replication*

Previous genetic and biochemical studies have demonstrated that the λ *cI* repressor blocks the initiation of λ DNA replication *in vivo* (Furth and Wickner, 1983) and in a crude *in vitro* system (McMacken *et al.*, 1983; M.S.Wold and R.McMacken, unpublished data), even in the presence of all required replication proteins. This regulatory dominance arises from the ability of the repressor to prevent transcriptional activation of λ DNA replication (Dove *et al.*, 1969, 1971). Transcriptional activation, however, is not required for the initiation of λ DNA replication in the minimal system reconstituted with purified proteins (Mensa-Wilmot *et al.*, 1989). Thus, if the inhibitory effect of repressor on λ DNA replication observed *in vivo* is solely mediated through its capacity to block the initiation of transcription, it might be anticipated that the λ repressor would not impede λ DNA replication in the minimal purified protein system.

This supposition is verified by the data presented in Table IV. The λ *cI* repressor did not inhibit λ DNA replication in the minimal system even when as many as 50 repressor dimers were added per template molecule of *ori*λ plasmid (Table IV, line 2). This represents a concentration of repressor that is approximately twice the concentration of repressor in a λ lysogen (Johnson *et al.*, 1981). The insensitivity of the minimal λ replication system to the λ repressor differs strikingly from the strongly inhibitory effect of repressor on λ DNA replication in a crude *in vitro* system. Replication of pRLM4 DNA, the same *ori*λ plasmid used in the studies reported here, is suppressed ~75% by supplementation of the crude system with 20 dimers of repressor per template circle (McMacken *et al.*, 1983).

Addition of HU and RNA polymerase to the mixture of purified proteins required for λ DNA replication enhanced the physiological nature of the *in vitro* system. In this system, as *in vivo*, replication of *ori*λ DNA depends on transcription of the template DNA. Since the λ repressor blocks initiation of transcription from the λ p_R promoter, which is the closest strong promoter to the *ori*λ sequence present on the template (Figure 1), initiation of DNA replication at *ori*λ in the coupled transcription-replication system may become subject to regulation by the *cI* repressor. When the *in vitro* system contains both HU and RNA polymerase, supplemen-

Table IV. Effect of the λ *cI* repressor on λ DNA replication *in vitro*

	Protein added			DNA synthesis (pmol)
	cI	HU	RNAP[a]	
1	−	−	−	260
2	+	−	−	320
3	+	−	+	280
4	−	+	−	4
5	+	+	−	2
6	−	+	+	170
7	+	+	+	86

The standard *ori*λ replication assay was modified. A series of reaction mixtures (10 μl), each containing 40 mM Hepes−KOH, pH 7.8, 11 mM magnesium acetate, 50 mM KCl, pRLM4 DNA (215 ng), and, as indicated, the λ *cI* repressor (142 ng), were assembled. All mixtures were incubated at 30°C for 15 min, chilled to 0°C and then supplemented with a mixture (20 μl) containing rNTPs (CTP, GTP and UTP, each at a concentration of 833 μM) and standard amounts of each of the components present in the standard *ori*λ replication assay (Materials and methods). HU protein (200 ng) and RNA polymerase (1.6 μg) were added as indicated. DNA synthesis was measured after a 45 min incubation at 30°C.
[a]RNAP, RNA polymerase.

Table V. Prior formation of an *ori*λ:O−P−DnaB nucleoprotein structure bypasses the inhibitory effect of HU on initiation of λ DNA replication

	Proteins added		Third-stage DNA synthesis (pmol)
	Stage 1	Stage 2	
1	λ mix	none	180
2	λ mix, HU	none	15
3	HU	λ mix	7
4	λ mix	HU	191
5	λ O	λ mix, HU	4
6	λ O, λ P	λ mix, HU	46
7	λ O, λ P, DnaB	λ mix, HU	151

Each reaction mixture was subjected to three sequential incubations. Stage 1 reaction mixtures (17 μl final volume) contained 70 mM Hepes−KOH, pH 7.6, 17 mM magnesium acetate, 7 mM ATP, bovine serum albumin (80 μg/ml), 264 ng pRLM4 DNA and the indicated proteins in the following amounts: HU, 100 ng; λ O, 195 ng; λ P, 100 ng; DnaB, 175 ng. λ mix contained λ O (195 ng), λ P (100 ng), DnaB (175 ng), DnaJ (50 ng), DnaK (3.6 μg), SSB (540 ng), primase (100 ng) and DNA gyrase (230 ng GyrA subunit and 240 ng GyrB subunit). Once assembled, each stage 1 mixture was incubated for 10 min at 30°C and then chilled to 0°C. Stage 2 components were added as indicated (final volume 27 μl). The composition of the λ mix was as described for the stage 1 addition, except that protein components previously added during the first stage were omitted from the mixture. A second-stage incubation was performed (10 min at 30°C) and the mixtures were again chilled to 0°C. Each mixture was supplemented with 80 ng of DNA polymerase holoenzyme and ^{3}H-labeled dNTPs as previously described (Mensa-Wilmot *et al.*, 1989) (33 μl final volume). DNA synthesis was measured after a third-stage incubation (5 min at 30°C).

tation of the system with λ repressor does in fact bring about a two-fold reduction in the level of DNA synthesis (Table IV, lines 6 and 7). Complete repression of DNA synthesis under these conditions would not be expected. For example, there is a low but significant level of *ori*λ-independent DNA synthesis mediated by RNA polymerase transcription (Table III). Moreover, the absence of specific transcription termination factors from the *in vitro* system may permit more distant repressor-insensitive promoters located on the circular template to direct transcription across the *ori*λ region. This most likely would result in some transcriptional activation of the initiation of λ DNA replication regardless of the presence of λ *cI* repressor.

*Assembly of a prepriming nucleoprotein complex containing the E.coli DnaB helicase at ori*λ *circumvents the regulation of* λ *DNA replication by HU protein*

Initiation of DNA replication at the phage λ replication origin proceeds through an ordered series of protein addition steps at *ori*λ prior to DNA unwinding, priming and DNA synthesis (Dodson *et al.*, 1985, 1986, 1989; Alfano and McMacken, 1989). Initially the λ O initiator binds in multiple copies to repeating sequences present at *ori*λ (Tsurimoto and Matsubara, 1981) and self-associates to form a specific nucleoprotein structure, termed an O-some, in which the origin DNA is inferred to be folded or wound (Dodson *et al.*, 1985). The O-some in turn serves as the locus for the addition of the λ P protein and the *E.coli* DnaB protein. These protein−protein interactions result in the formation of a larger nucleoprotein structure at *ori*λ (Dodson *et al.*, 1985), a complex that contains O, P and DnaB (Alfano and McMacken, 1989; Dodson *et al.*, 1989).

We wished to learn more about how HU exerts its regulatory effect on the initiation of λ DNA replication. We examined the possibility that the histone-like HU protein interferes with the assembly of one or more of the prepriming nucleoprotein structures formed at *ori*λ. To investigate this idea we varied the order in which HU and required replication proteins were added to the *ori*λ plasmid template. Specific nucleoprotein prepriming structures were allowed to form at *ori*λ prior to the addition of HU to the reaction mixture. We then determined if the preformed prepriming complex was functionally active in the presence of HU. Thus, each specific nucleoprotein structure was incubated with HU and the remainder of the proteins and factors required for initiation of λ DNA replication, and the amount of DNA synthesized was determined (Table V).

If the *ori*λ plasmid DNA was first incubated in the minimal λ replication system, in the absence of DNA polymerase III and deoxyribonucleoside triphosphates (dNTPs), to produce prereplicative intermediates, subsequent incubation of these intermediates with HU did not affect their potential to be converted to replication products upon addition of DNA polymerase III and dNTPs (Table V, lines 1 and 4). But, if HU was permitted to bind to the *ori*λ template prior to the addition of the proteins required for λ DNA replication, only limited DNA synthesis was obtained (Table V, line 3). These results indicate that the inhibitory effect of HU on λ DNA replication is not imposed through a block on the propagation of replication forks. Instead, the interaction of HU with the *ori*λ plasmid template apparently interferes with an earlier step in the reaction such as DNA unwinding, priming, or the proper assembly or function of nucleoprotein structures required for the initiation of λ DNA synthesis.

Additional experiments were performed to identify the primary step at which HU exerts its inhibitory effect on λ DNA replication. HU apparently acts at a stage after the formation of an O-some at *ori*λ. Incubation of the *ori*λ plasmid template with λ O protein prior to the addition of HU protein to the *in vitro* system did not circumvent HU-mediated repression of λ DNA replication (Table V, line 5). In contrast, once an O−P−DnaB nucleoprotein complex

is assembled at the λ origin, subsequent stages in the replication of *ori*λ plasmids proceed unimpeded by the presence of HU protein (Table V, line 7). Thus, HU may interfere with the prepriming step of λ DNA replication in which an O-some is converted to a functional *ori*λ:O−P−DnaB complex.

Discussion

The initiation of bacteriophage λ DNA replication *in vivo* is distinguished by its nearly complete dependence on transcriptional events at or near *ori*λ (Dove *et al.*, 1969, 1971). This strict coupling of transcription and initiation of DNA replication at *ori*λ permits the phage *cI* repressor to directly block λ DNA replication (Thomas and Bertani, 1964; Green *et al.*, 1967) by restricting transcription from the λ p_R promoter (Figure 1), the only viral promoter capable of directing transcription across *ori*λ.

A diverse group of prokaryotic (e.g. *E.coli*, phage T7, λ and plasmid ColE1) and eukaryotic (e.g. yeast, SV40, polyoma virus, adenovirus, Epstein-Barr virus, bovine papilloma virus and mitochondrial DNA) replicons depend on transcription or on transcriptional activating proteins or elements to facilitate initiation of DNA replication *in vivo* (Kornberg, 1980; Zyskind and Smith, 1986; McMacken *et al.*, 1987; DePamphilis, 1988). What roles might transcription or transcriptional proteins serve in the initiation of chromosomal DNA replication? Transcription could supply a primer transcript needed for DNA chain elongation. Alternatively, transcription could activate replication by altering the structure of the replication origin, for example, by unwinding the duplex DNA to allow assembly of elongation machinery. Or, the origin *in vivo* may be compacted into an inactive form by chromosomal binding proteins and transcription or transcriptional activating proteins could serve to increase the accessibility of the origin to required replication proteins.

In the limited number of systems where the molecular role of RNA polymerase transcription in DNA replication has been determined, it has usually been found that an RNA transcript was used to prime leading strand DNA chain elongation. This mechanism was initially demonstrated for the conversion of bacteriophage M13 viral strands to the duplex replicative form (Wickner *et al.*, 1972) and later shown to be operative in the initiation of both ColE1 plasmid and bacteriophage T7 DNA replication (Itoh and Tomizawa, 1980; Fuller and Richardson, 1985). In contrast, a direct role for RNA polymerase-mediated priming of λ and *E.coli* DNA replication is not likely, despite the strict dependence of the initiation of λ and *E.coli* chromosomal DNA replication on transcription *in vivo*. Extensive DNA synthesis dependent on *ori*λ or *oriC* can be obtained in reconstituted multiprotein replication systems in the absence of any detectable RNA polymerase activity (Mensa-Wilmot *et al.*, 1989; Funnell *et al.*, 1986).

The surprising discovery that RNA polymerase transcription is not required for the initiation of λ DNA replication in a system composed of nine purified λ and *E.coli* replication proteins (Mensa-Wilmot *et al.*, 1989) led us to search for cellular factors that could restore the physiological linkage between transcription and replication. This search led to the identification of *E.coli* HU protein, a small, abundant, histone-like protein, as the predominant cellular protein which has the capacity to reinstate the transcriptional regulation of the initiation of λ DNA replication. IHF, a histone-like relative of HU, was found to have similar effects on λ DNA replication *in vitro*. However, it is unlikely that IHF plays a major role in regulating λ DNA replication *in vivo* in wild-type *E.coli*, given its low intracellular concentration (estimated to be 4- to 10-fold lower than that of HU). Due to the complex nature of the assay we used to identify transcription-sensitive inhibitors of λ DNA replication *in vitro*, we cannot exclude the possibility that additional *E.coli* proteins augment the inhibition mediated by HU protein or even substitute for it altogether.

HU is a heterotypic dimer composed of closely related subunits, HUα and HUβ, coded by the *hupA* and *hupB* genes. It can wrap and compact DNA into nucleosome-like structures (Berthold and Geider, 1976; Rouvière-Yaniv *et al.*, 1979; Broyles and Pettijohn, 1986) and it is known to be the primary protein constituent of the bacterial nucleoid (Varshavsky *et al.*, 1977; Rouvière-Yaniv, 1978). Although the precise role of HU is not known, the importance of this protein to cellular physiology is underscored by the finding that closely related proteins are present in all eubacterial and archaebacterial species that have thus far been examined (Drlica and Rouvière-Yaniv, 1987). HU was first isolated as a protein that stimulated the transcription of bacteriophage λ DNA by *E.coli* RNA polymerase *in vitro* (Rouvière-Yaniv and Gros, 1975). Other *in vitro* biochemical assays indicate that HU stimulates initiation of DNA replication at the *E.coli* origin (Dixon and Kornberg, 1984), and that it is involved in replicative transposition of phage Mu (Craigie *et al.*, 1985) and in site-specific DNA inversion of the *hin* gene of *Salmonella typhimurium* (Johnson *et al.*, 1986).

In the absence of genetic verification, can we be certain that the capacity of HU to block the initiation of λ DNA replication *in vitro* is also manifested *in vivo* when transcription of the origin region is limiting? Two properties of the HU-mediated inhibition of λ DNA replication in the purified protein system are consistent with a physiological role for HU in the regulation of λ DNA replication *in vivo*. First, the presence of HU in the reconstituted λ replication system restores the physiological linkage between transcription and the initiation of λ DNA replication. Second, although the λ repressor directly inhibits phage DNA replication *in vivo*, the simultaneous presence of HU is needed to enable the λ repressor to block initiation of λ DNA replication *in vitro* (Table IV).

Our data suggest that once a nucleoprotein complex containing the λ O and P proteins and the *E.coli* DnaB helicase is assembled at *ori*λ, HU loses its potency as an inhibitor (Table V). λ DNA replication proceeds unimpeded in the presence of the histone-like HU protein, even in the absence of transcription, if the *ori*λ:O−P−DnaB nucleoprotein structure is allowed to form prior to the addition of HU to the reaction mixture. This may mean that HU acts *in vitro* to block the assembly or activity of an O−P−DnaB protein complex at *ori*λ. This interpretation would be consistent with two previous reports that RNA polymerase may function at a similar step during the transcriptional activation of the initiation of λ DNA replication. Genetic evidence suggests that a functional interaction of RNA polymerase with DnaB and P is required for the initiation of λ DNA replication *in vivo* (McKinney and Wechsler, 1983). Additionally, recent studies of the role of transcrip-

tion in the replication of λ DNA in a crude *in vitro* system, a system that undoubtedly contains HU, indicate that an early prepriming step depends on RNA polymerase, DnaB and the λ O and P proteins (Yamamoto *et al.*, 1987).

Initiation of DNA replication at the *E.coli* chromosomal origin, *oriC*, is analogous in several respects to the initiation of λ DNA replication. In both systems nucleosomal structures, formed by specific initiator proteins at the genetically defined replication origin, function as loci for the transfer of DnaB helicase onto the template (Fuller *et al.*, 1984; Dodson *et al.*, 1985, 1986, 1989; LeBowitz *et al.*, 1985; Baker *et al.*, 1986; Funnell *et al.*, 1987; Alfano and McMacken, 1988, 1989). Furthermore, following initiation of DNA synthesis *in vitro*, the propagation of replication forks from *oriC* and *ori*λ is apparently carried out by the same set of *E.coli* replication proteins (LeBowitz and McMacken, 1986; Baker *et al.*, 1986; Funnell *et al.*, 1986; Alfano and McMacken, 1988, 1989; Mensa-Wilmot *et al.*, 1989).

The similarity of the two systems may even extend to the biochemical mechanisms used to regulate the frequency of initiation of DNA replication. Numerous physiological and genetic studies indicate that the initiation of a round of *E.coli* DNA replication *in vivo*, as for phage λ, depends on and may be regulated by transcription by the bacterial RNA polymerase (Zyskind and Smith, 1986; McMacken *et al.*, 1987; von Meyenburg and Hansen, 1987). In addition, as demonstrated here for λ DNA replication, the *E.coli* HU protein causes a reconstituted *oriC* replication system to become dependent on RNA polymerase transcription when this bacterial histone-like protein is present in amounts sufficient to coat 30–50% of the input *oriC* template (Ogawa *et al.*, 1985). Determining the mechanism of HU protein action in the *oriC* system is complicated by the fact that HU protein exerts two additional effects that have not been observed in the λ DNA replication system. HU protein at low concentrations activates *oriC* DNA replication several fold (Ogawa *et al.*, 1985) and at higher concentrations enhances the template specificity of the multiprotein *oriC* replication system (Kaguni and Kornberg, 1984).

What plausible mechanisms could account for the capacity of HU to block the initiation of λ DNA replication and yet also be generally applicable to other replication systems, such as the one for *oriC* plasmids? Since initiation of DNA replication at both *ori*λ and *oriC* are strongly dependent on the presence of negative superhelicity in the template DNA (Alfano and McMacken, 1988; Baker and Kornberg, 1988), one obvious possibility is that the binding of multiple HU molecules to the template DNA restrains sufficient negative supercoils to block the initiation of DNA replication (Baker and Kornberg, 1988). In the reconstituted λ replication system, ~75 ng of HU is required to obtain 50% inhibition of the initiation of λ DNA replication. It can be estimated (Drlica and Rouvière-Yaniv, 1987) that this amount of HU would under ideal conditions restrain only six or seven of the ~37 negative supercoils that are present in the *ori*λ plasmid template. Furthermore, under standard *ori*λ replication conditions, 200 ng of HU (i.e. a 1:1 weight ratio of HU to DNA) restrained just two supercoils, as judged by a typical assay for protein wrapping of DNA using DNA topoisomerase (K.Mensa-Wilmot and R.McMacken, unpublished data). It remains to be determined, however, if such relatively small reductions in the superhelicity of the template block initiation of λ DNA replication in the minimal *in vitro* system.

Materials and methods

Bacterial strains and plasmids

E.coli K12 strain C600 was described previously (Wold *et al.*, 1982). Plasmids pRLM4, pRLM5, M13mp8 RF and M13*oriC*26 RF have been previously described (Messing and Vieira, 1982; Kaguni *et al.*, 1979; Wold *et al.*, 1982).

Materials

Reagents and their sources were: calf thymus DNA, egg white lysozyme and streptomycin sulfate, Sigma Chemical Co.; DEAE–Sephadex, Pharmacia; cellulose powder CF11, Whatman; Centricon microconcentrators and Diaflo ultrafiltration membranes, Amicon. DNA–cellulose, containing 1.2 mg of DNA per ml of packed resin, was prepared by mixing native calf thymus DNA with cellulose (Alberts *et al.*, 1968). The sources of other materials were described previously (Wold *et al.*, 1982; LeBowitz and McMacken, 1984, 1986; Mensa-Wilmot *et al.*, 1989).

Buffers

TE buffer is 10 mM Tris–HCl, pH 8.0, 1 mM EDTA; buffer A is 25 mM Hepes–KOH, pH 7.6, 1 mM EDTA; buffer B is 25 mM Hepes–KOH, pH 7.6, 0.1 mM EDTA and 2 mM dithiothreitol (DTT); buffer C is 20 mM Tris–HCl, pH 7.8 (25°C), 10% (w/v) glycerol, 1 mM EDTA and 1 mM DTT; buffer D is 20 mM Tris–HCl, pH 7.0, 10% (w/v) glycerol, 1 mM EDTA, 1 mM DTT and 50 mM NaCl.

Proteins and protein analysis

Purified HU protein (Losso *et al.*, 1986), the generous gift of C.Gualerzi (University of Camerino), was supplied as individual preparations of homogeneous α and β subunits (proteins NS2 and NS1, respectively). Unless stated otherwise, native HU protein was reconstituted for each experiment by mixing equimolar amounts of the α and β polypeptide chains together. For certain experiments homogeneous native HU protein was used. Native HU protein was purified using an unpublished protocol developed and provided by Dr Kenneth Marians (Memorial Sloan-Kettering Cancer Institute, New York). All bacteriophage λ and *E.coli* replication proteins were as described previously (Mensa-Wilmot *et al.*, 1989). Homogeneous preparations of IHF and λ *cI* repressor were donated by H.Nash (National Institutes of Health) and C.Pabo (The Johns Hopkins University), respectively. Antibody preparations directed against native HU protein or NS1 protein (HUβ polypeptide) were provided by Drs J.Rouvière-Yaniv (Institut Pasteur) and C.Gualerzi, respectively. Immunological evaluations of purified proteins were carried out by an immunoblot analysis. Individual protein samples (200 ng) were spotted on nitrocellulose, incubated with specific antibodies, and visualized by a modification of a published protocol (Towbin *et al.*, 1979). Protein concentrations were determined by the method of Lowry (Lowry *et al.*, 1951), using bovine serum albumin as the standard.

Assay of λ DNA replication

λ DNA replication was measured using the nine-protein reconstitution assay for *ori*λ plasmid (λ*dv* plasmid) DNA replication described previously (Mensa-Wilmot *et al.*, 1989). Plasmid pRLM4 DNA (Wold *et al.*, 1982) was used as the template DNA in this minimal λ replication system.

Assay for a transcription-sensitive inhibitor of λ DNA replication

Preliminary experiments indicated that soluble crude extracts prepared from *E.coli* contained factors that diminished the capacity of the minimal λ replication system to support the replication of *ori*λ plasmid DNA. With relatively crude protein fractions, the maximal replication inhibition depended on preincubation of the protein fraction with the plasmid template. The assay described below was designed to identify factors whose inhibitory effects on λ DNA replication could be counteracted by transcription of the template by *E.coli* RNA polymerase.

A protein fraction containing inhibitory factors was first titrated to determine the maximal amount of protein that could be directly added to the minimal λ replication system without significantly altering the level of DNA synthesis obtained in the standard *ori*λ replication reaction. The amount of protein so determined was increased 3.3-fold and added to 720 ng (3.3-fold the standard amount of template) of supercoiled pRLM4 DNA in 10 μl (final volume) of preincubation solution (40 mM Hepes–KOH, pH 7.6, 11 mM magnesium acetate, 3 mM ATP, 500 μM each CTP, GTP and UTP,

0.83 mg/ml *E.coli* tRNA, 54 mM creatine phosphate, 100 μg/ml creatine kinase, 50 μg/ml bovine serum albumin, 6.7 μg/ml gyrase A subunit and 6.7 μg/ml gyrase B subunit). Each mixture was incubated for 10 min at 30°C. Subsequently, a 3 μl portion of the preincubation mixture was added to the standard reconstitution mixture developed for the replication of *ori*λ plasmid DNA (Mensa-Wilmot *et al.*, 1989), except that template DNA was omitted from the latter mixture. The combined mixture (denoted replication assay 1) was incubated for 30 min at 30°C and the incorporation of labeled deoxynucleotide into acid-insoluble material was determined as previously described (Mensa-Wilmot *et al.*, 1989). A second λ replication reaction was performed to determine if any inhibition produced by the preincubation mixture could be counteracted by transcription of the pRLM4 template DNA with RNA polymerase. In this control reaction (denoted replication assay 2), a second 3 μl portion of the aforementioned preincubation mixture was added together with *E.coli* RNA polymerase (1.6 μg) and with CTP, GTP, and UTP (each at a final concentration of 500 μM) to the standard *ori*λ replication mixture (except that pRLM4 template DNA was omitted from this latter mixture). The complete mixture was incubated for 30 min at 30°C and the amount of DNA synthesis was determined as described above. A protein fraction was considered to contain possible regulatory activity when it both (i) strongly inhibited λ DNA replication in replication assay 1 and (ii) exhibited greatly diminished inhibitory capacity in the presence of active transcription in replication assay 2.

Once the λ replication inhibitor had been substantially purified (i.e. after the DNA–cellulose chromatography step described below), it was no longer necessary to preincubate active protein fractions with *ori*λ DNA in order to obtain optimal inhibition of DNA synthesis. Instead replication inhibitory activity was assessed simply by adding protein fractions directly to the reconstituted λ replication system (Mensa-Wilmot *et al.*, 1989) and incubated at 30°C either in the presence or absence of RNA polymerase and rNTPs.

Gel electrophoresis

Protein samples were electrophoresed in the presence of sodium dodecyl sulfate in a 10–15% gradient polyacrylamide gel (Laemmli, 1970).

Isolation of an inhibitor of oriλ plasmid DNA replication

Unless stated otherwise, all steps were performed at 4°C.

Cell growth and lysis. E.coli C600 was grown at 37°C in AZ broth (Ueda *et al.*, 1978) to mid-log phase (7×10^8 cells/ml) in a New Brunswick FM250 fermentor. Cells were rapidly chilled to 4°C and harvested in a CEPA Z-80 continuous flow centrifuge. The cell pellet was resuspended in an equal volume of buffer A, frozen in liquid nitrogen and stored at −70°C. Cells (50 g) were lysed by freeze–thaw cycles as described previously (Fuller *et al.*, 1981), except that T4 lysozyme was omitted from the lysis mixture. Lysates were clarified by high-speed centrifugation (150 000 *g* for 30 min at 2°C) to generate Fraction I (107 ml).

Streptomycin sulfate precipitation. Fraction I was diluted with an equal volume of buffer A containing 2 mM DTT. Streptomycin sulfate (10% w/v) was added dropwise with stirring to a final concentration of 2%. After an additional 15 min of stirring, the suspension was centrifuged at 12 000 *g* for 15 min and the resulting supernatant fraction was discarded. The pellet, containing nucleic acids and nucleoproteins, was suspended in 20 ml of buffer B containing 0.3 M KCl and stirred slowly for 1 h. This suspension was centrifuged at 12 000 *g* for 20 min and the supernatant was saved. The pellet was extracted a second time with 20 ml of buffer B containing 0.3 M KCl and centrifuged as described above. The two supernatant fractions were combined to yield fraction II (57 ml).

Ammonium sulfate fractionation. Ammonium sulfate (0.28 g/ml Fraction II) was slowly added to Fraction II with gentle stirring. After 30 min of additional stirring, precipitated protein was removed by centrifugation (20 min at 27 000 *g*). Additional ammonium sulfate was slowly added to the supernatant fraction (0.12 g ammonium sulfate/ml) and after 30 min the precipitate was collected by centrifugation. The well-drained pellet was suspended in 21 ml of buffer C (Fraction III: 26.7 ml; 640 mg protein).

DEAE–Sephacel chromatography. A 20 ml portion of Fraction III was dialyzed for 12 h against 4 l of buffer C containing 50 mM NaCl and loaded onto a 3 × 10 cm column of DEAE–Sephacel that had been equilibrated in the same buffer. The column was washed with 300 ml of buffer C + 50 mM NaCl and the inhibitor of λ DNA replication was eluted with 300 ml of buffer C + 200 mM NaCl. Fractions containing significant levels of protein were pooled (Fraction IV; 240 ml; 56 mg protein).

DNA–cellulose chromatography. Fraction IV protein was applied to a 4 × 4 cm double stranded DNA–cellulose column that had been equilibrated in buffer C + 200 mM NaCl. The column was washed with 150 ml of buffer C + 200 mM NaCl and bound protein was eluted with a 400 ml linear gradient of 200–400 mM NaCl in buffer C. Fractions of 8 ml were collected. Fractions containing protein that inhibited λ DNA replication were centered around 0.36 M NaCl. The peak fractions were pooled (40 ml), concentrated by ultrafiltration to 5 ml using a Diaflo PM10 membrane and dialyzed for 12 h against 1 l of buffer D. The dialyzed protein sample was applied to a 0.5 ml DEAE–Sephacel column equilibrated in buffer D. The column was washed with 1 ml of buffer D and the inhibitor of λ DNA replication was eluted wtih 1.5 ml of buffer D containing 200 mM NaCl (Fraction V; 160 μg protein).

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